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Table of Contents

Title: Validation of a Pre-Clinical Model for the Investigation of Menarcheal Age on Breast Cancer Risk

Beginning Date 09-01-2005, with a one year extension granted for an ending date of 08-31-2007. A one year extension was granted because the PI, Dr. Pepper Schedin, moved her lab from AMC Cancer Research Center in Denver, CO to the University of Colorado Health Sciences Center. This move resulted in a 6 month delay in finalizing the grant award. As a result, work on this grant was delayed until June 2006 and thus this report represents progress made in the last 6 months.

Introduction: Individuals with late menarche have breast cancer rates that are approximately 2 fold lower than individuals with early menarche, however the mechanism of protection is unknown. Two theories dominate; the model of breast tissue aging proposes that cumulative lifetime exposure to circulating ovarian hormones determines risk. The second theory suggests that early menarche is associated with persistent qualitative differences in the hormone axis or in the gland itself. **Purpose:** Determine whether an extensively utilized preclinical model for human breast cancer, the SD rat model, demonstrates the relationship between age of sexual maturation and mammary cancer risk that is observed in humans. **Aims: 1)** Determine the relationship between age at vaginal opening (VO), a marker for ovarian function, and susceptibility to MNU-induced mammary cancer and **2)** investigate the hypothesis that early sexual maturation confers increased breast cancer risk by persistently altering systemic hormone levels and/or by altering the response of the gland to subsequent hormone stimulation. **Methods:** Sexually immature female SD rats, monitored for VO, will be separated into Gp 1, the first 25% of rats to reach VO and Gp 2, the last 25% to reach VO. Effect of age of VO on estrous cycling, mammotrophic hormone levels, ER and PR expression in the mammary gland, exogenous hormone stimulation, and susceptibility to mammary carcinogenesis will be determined. **Relevance**: Once characterized, this pre-clinical model can be utilized by the breast cancer community to investigate the mechanism(s) by which early menarche increases breast cancer risk.

Body: Describes the research accomplishments associated with each task in the approved statement of work.

Task 1a. Identify Sprague-Dawley female rats with early and late onset of sexual maturation.

Months 1-2. Rationale: As with humans, outbred Sprague Dawley (SD) female rats demonstrate a bell-shaped curve relationship between age and maturation of the ovaries. Thus, rats can be segregated into groups of early and late onset of sexual maturation. Vaginal opening will be used as the marker for onset of menarche because VO is an estrogen dependent process, thus is dependent on ovarian function. Most rats have one complete estrous cycle within10 days of VO. Two hundred 21 day of age (p21) female SD rats (with precise date of birth known) will be fed AIN-93G diet, a defined diet optimized for rapidly growing young rats. Starting at p26, the rats will be evaluated daily for onset of vaginal opening (VO), by visual inspection. The first 50 rats to reach VO will be segregated into the early-onset group (Group 1) and the last 50 rats to reach VO will be segregated into the late-onset group (Group 2). Based on previous experience, it is anticipated that 50 rats will reach sexual maturation by p30, (Group 1), 100 will reach maturity between p30 and p35 and will not be utilized in this study, and 50 will have late onset of sexual maturation, occurring at or later than p36 (Group 2).

Status: Completed

Results: Two hundred 20 day of age female SD rats (p21) were obtained from Sprague-Dawley Harlan and monitored daily by visual inspection for vaginal opening. Thirty nine rats had vaginal opening between 28 and 30 days of age, 123 had vaginal opening between 31and 33 days of age and 38 had vaginal opening at 34 days of age or later. The distribution of age at vaginal opening is shown in **Figure 1**. For this study, we utilized rats with vaginal opening at 30 days of age or younger for the early VO group and those with vaginal opening of 34 days of age or later as the late VO group.

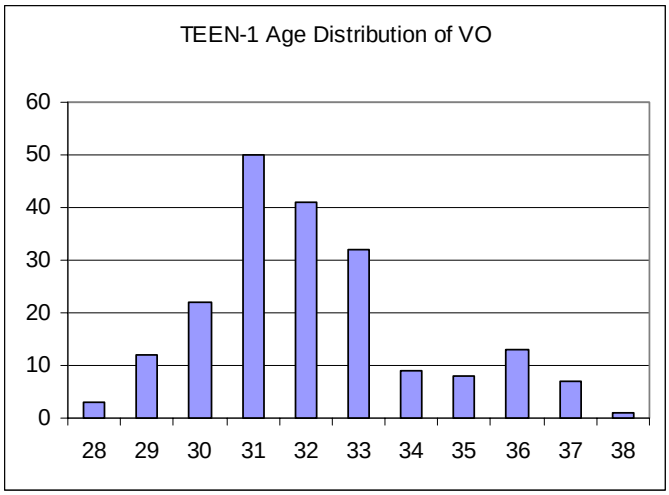


Fig. 1 Distribution of age at vaginal opening (VO) in 200 female SD rats. Rats with VO of 30 days and younger were assigned to the early VO group and rats with a VO of 34 days of age and later to the late VO group.

Fig. 1

Unexpected Problems Encountered: Based on previous studies, we had anticipated obtaining a wider spread in age at VO than was found in this current study. One reason for the discrepancy may be that previous studies were performed using non-ventilated cages whereas this current study was performed using ventilated cages. We have discovered significance differences in estrous cycling and breeding habits between rats in the two different caging environs, thus ventilated caging may have influenced age at VO. As a result, we had significantly fewer rats in the early VO and late VO groups than anticipated (39 and 38 per group compared to an expected 50 per group). Thus, we decided to focus our efforts on Aim 2 of the grant, as we did not have enough animals to carry out the carcinogenesis study proposed in Aim 1.

Task 1b. Determine if there is a relationship between 1) body weight and age at VO and 2) age at VO and adult body weight. Rationale: In humans, correlations exist between body mass index in childhood and age of sexual maturation and adult body weight, however body mass index alone does not account for early onset of puberty. Starting at p21, body weights will be taken twice weekly to study end to characterize these relationships in the rat.

Status: Completed

Results: The relationship between body weight and onset of sexual maturation observed was complex. In **Figure 2**, it can be seen that overall, the rats that had early VO were as a group heavier than the rats that had late VO. This data models the relationship observed between onset of puberty and body weight in girls. Of note, and as can be seen in **Figure 2**, the difference in body weight between groups was observed early, by 28 days of age, and persisted with age but did not increase in magnitude with time.

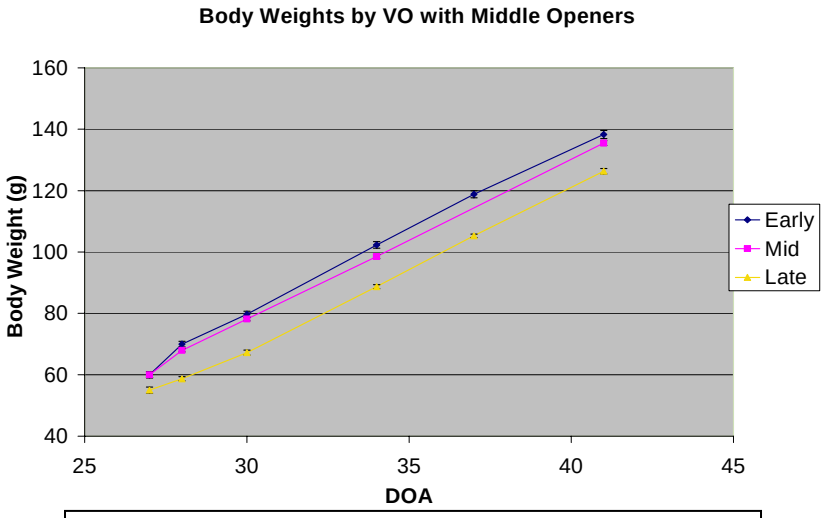
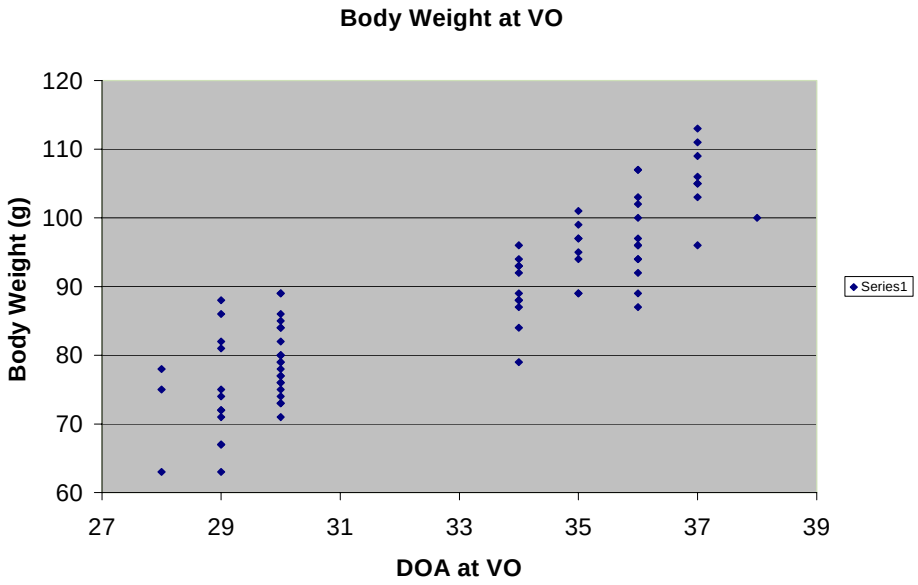


Fig. 2 Rats with early VO had, as a group, higher body weights than rats with late VO.

In **Figure 3**, the weight of each rat on the day of VO is shown. It can be seen that overall, animals in the early VO group reached sexual maturation at a much lower body weight than rats in the late VO group. These data suggest that body weight alone does not account for entry into VO, but rather a combination of body weight in conjunction with a 'permissive' endocrine status/profile is likely to be responsible for onset of VO.

Fig. 3 Body weight at time of VO was lower in the early VO group than in the late VO group.



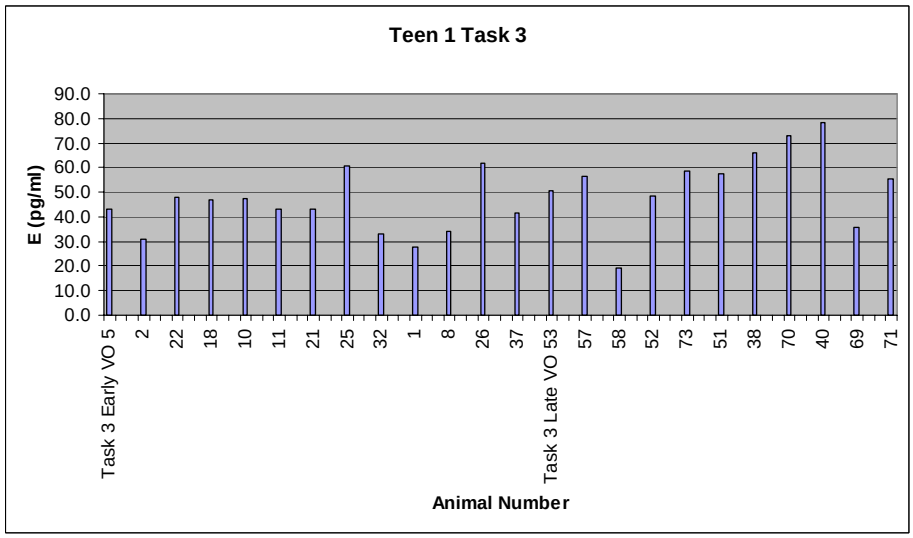
Task 2. Determine whether age at VO alters circulating 17-β-estradiol, progesterone and IGF-1 levels in the mature rat. **Months 3-5.** Rationale: Because of the predominance of data demonstrating a strong positive correlation between cumulative estrogen exposure and breast cancer risk, it has been hypothesized that women with elevated levels of circulating ovarian hormones (and more recently IGF-1) would have increased risk for breast cancer. Similarly, the elevated risk of breast cancer associated with early menarche may be due to higher circulating mammotrophic hormone levels in women with early compared to late menarche. The question of whether circulating hormone levels correlate with breast cancer risk has been repeatedly addressed, with mostly negative results. However, in premenopausal women, it is difficult to control for the day in the menstrual cycle from which blood is collected and further, to control for anovulatory cycles. Thus, the reported negative data may be confounded by these uncontrolled variables. In the rat model, we have the ability to carefully control for both cycle regularity and stage; thus, it will be possible to more accurately determine whether a correlation exists between age at VO and circulating ovarian hormone levels in the sexually mature rat.
Task 2a. Blood collection for hormone analyses. At p63, 25 rats per group will be evaluated daily for stage of estrous by cervical lavage. The question of whether age at VO influences the length or regularity of the cycles will be determined over a two week period. To control for variation in circulating hormone levels that could be due to either length of cycle or irregularity of cycle, only rats identified to have regular four day cycles will be selected for further evaluation. Based on previous experience, we anticipate identifying a minimum of 10 rats per group with regular four day cycles. One milliliter of blood per rat will be obtained by orbital eye bleed at two stages of the estrous cycle; correspond to low and high circulating ovarian hormone levels, respectively. Circulating hormone levels are lowest during diestrus 1 of the cycle and blood samples for this stage will be collected at 12:00 pm. Conversely, blood will be collected at 6:00 pm on day of proestrus, a stage and time of day that corresponds to the highest levels of estrogen and progesterone, as detection by RIA analyses.
Status: Animal Work Completed. We have obtained blood from regular cycling rats (all 5 day cyclers) during proestrus and diestrus 1 stages of their cycle in 14 rats with early VO and 12 rats with late VO.

Task 2b. ELISA analyses for estrogen, progesterone and IGF-1 levels. Immediately after collection, blood samples will be centrifuged at 1,200g for 10 min at 4°C and serum stored at -80°C until analyses. For estrogen, progesterone and IGF-1 level determination, each sample will be evaluated in triplicate using commercially

available ELISA kits, and levels quantified by comparison with known standards (Alpha Diagnostics, San Antonio, TX). Data will be evaluated using RIAAID software (Hazelton, WA).

Status: Analyses in Progress

Task 3 hormone analyses-new task. We have also collected blood from animals assigned to Task 3 (described below). For this study, blood was collected in the estrus phase of the cycle from 13 rats with early VO and 11 rats with late VO, and processed for sera and plasma. To date, these samples have been evaluated for estradiol by RIA. To our surprise, circulating estradiol levels were found to be higher in the rats with late VO compared to rats with early VO. **Figure 4** shows the results for each animal and figure 5 shows the average circulating estradiol levels per group.



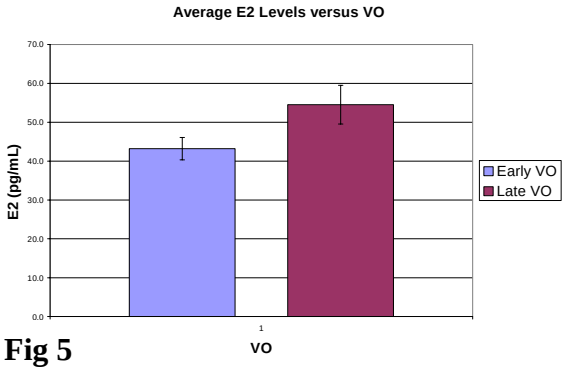


Fig 5

Fig 4 Circulating estradiol levels from individual rats with early and late VO and **Fig 5**, average estradiol levels per group. Late VO group average estradiol levels significantly higher than early VO group, p=0.03.

Task 3: Determine whether age at VO alters the number of mammary epithelial cells expressing ERα, ERβ, PRA and PRB. Months 6-8. Rationale: An alternative mechanism by which early age menarche increases risk for breast cancer is by persistently altering the number of mammary epithelial cells expressing hormone receptors or by altering the ratio of ERα/ERβ and PRA/PRB; alterations of which are observed in many human breast cancers. The number of MEC expressing steroid receptors will be determined by IHC. For these IHC analyses, 5 rats per group will be euthanized during the estrus phase of the estrous cycle (stage of cycle maximally responsive to hormonal stimulation) and mammary tissue adjacent to the lymph node chain in mammary gland #4 harvested to minimize differences in gland morphology due to proximal/distal location in the gland. Stage of estrous will be confirmed by histological evaluation of cervical tissue. Mammary tissue will be fixed for 24hr in 10% neutral buffered formalin, embedded in paraffin and cut into 5-µm sections. Our research team has ERα, ERβ, PRA and PRB specific antibodies that have been optimized and validated for formalin fixed rat tissue.

Status: Animal work is completed and all tissues have been processed to paraffin-embedded block stage.
Results: IHC analysis in progress

Task 4. Determine whether age at VO alters response of mature gland to hormone stimulation. Months 6-8

Task 4a. Determine whether age at VO alters the percent of MECs that undergo proliferation in response to hormonal stimulation. Rationale: In response to the cyclic hormonal stimulation, approximately 15% of MEC per cycle undergo a round of proliferation. One mechanism which could account for the increased risk in breast cancer risk with early menarche is that a higher proportion of MEC respond to the circulating E&P by undergoing proliferation. To address this question, the effect of VO on the proliferative index of mammary epithelium challenged with the mitogenic hormones estrogen and progesterone will be determined. At p63, 5

rats per group will be ovariectomized. One week post-ovariectomy, rats will be challenged with 5 µg 17-β-estradiol and 1.5 mg progesterone. Twelve hours post hormone injection, animals will be treated with 50 mg BrdU/kg body weight by i.p. injection and animals sacrificed two hours later. Mammary tissue, collected from gland #4 and controlled for proximal/distal location within the gland, will be fixed in methacarn, a fixative compatible for immunohistochemical detection of BrdU using anti BrdU antibody from Becton Dickinson. Ten randomly chosen fields will be selected per gland, photographed at 400X and the percentage of BrdU positive cells determined independently by two investigators using coded photographs. A minimum of 1000 epithelial cells per gland will be evaluated.
Status: Animal work is completed and all tissues have been processed to paraffin-embedded block stage.
Results: IHC analysis in progress

Task 4b. Evaluate the effect of VO on MEC apoptotic index. Rationale: In order to maintain gland homeostasis under the conditions of repeated cyclic ovarian hormone stimulation, the number of MECs that undergo apoptotic cell death is equaled to the number that proliferate during each cycle. A reduction in the number of cells susceptible to estrous cycle-dependent cell death would be anticipated to increase risk for carcinogenesis. To address the question of whether early VO results in fewer MEC undergoing cell death, the number of apoptotic cells in the mammary glands of rats described in Task 4a will be determined. Fragmented DNA in apoptotic cells will be labeled at 3'-OH ends in situ using the TUNEL assay with digoxigenin-labeled dUTP and detected immunohistochemically using anti-digoxigenin antibody following manufacturer's protocol (R&D Systems). Percent apoptotic cells will be evaluated as described for quantization of proliferating cells described in **Task 4a**.
Status: Animal work is completed and tissues have been processed to paraffin-embedded block stage.
Results: TUNEL analysis has not yet begun.

Task 4c. Determine whether age at VO influences mammary gland alveolar morphology.
Rationale: A correlation exists between the degree of mammary alveolar development and susceptibility to carcinogenesis. Further, exogenous progesterone increases alveologenesis and can increase risk for breast cancer in women. We will determine whether mammary gland alveolar development is altered by VO, using morphological criteria originally described by the Russos. Mammary glands will be processed to 5µm sections, stained with Hematoxylin and Eosin and alveologenesis quantified; no side branches will be scored as ductal, mammary glands with side branches ending in lobules composed of $\leq$ 5 acini per lobular will be scored as weakly-alveolar, those with $\leq$ 10 acini per lobule as moderately-alveolar and those with > 11 acini per lobule as highly alveolar.
Status: Animal work is completed and all tissues have been processed to paraffin-embedded block stage.
Results: Quantitative morphometry analysis in progress

Task 5. Determine whether age at VO alters response of gland to carcinogenic insult.
Months 3-12
At p70, all remaining rats (40 per group) will be injected i.p. with 50 mg MNU per kg body weight and tumor incidence, latency and multiplicity followed for 6 months by twice-weekly manual palpation**.** At necropsy, tumors will be harvested, weighed and processed for histological evaluation. Only confirmed adenocarcinomas will be included in statistical analyses for tumor latency, incidence, multiplicity and tumor burden.
Status: As originally proposed, it was anticipated that this carcinogenesis study could be accomplished along with the above described tasks from a starting population of 200 rats. However, considerably fewer animals segregated into the early and late VO groups than expected, thus this arm of the study has been delayed. Our strategy is to determine whether differences between the early and late VO groups are identified, as described in Task 2-4 prior to initiating the carcinogenesis study. Identifying biological differences in hormone signaling would justify the significant added expense of replicating the animal husbandry component of this grant.

Key Research Accomplishments:

1. Using the Sprague-Dawley female rat, we have determined that it is possible to segregate rats into those with early VO and late VO.
2. Have generated data that suggests that the relationship between body weight and age at VO is complex. Overall, the rats that had early VO were as a group heavier than the rats that had late VO. This data models the relationship observed between onset of puberty and body weight in girls. However, animals in the early VO group reached sexual maturation at a much lower body weight than rats in the late VO group. These data suggest that body weight alone does not account for entry into VO, but rather a combination of body weight in conjunction with a 'permissive' endocrine status/profile is likely to be responsible for onset of VO.
3. We have found that rats with late VO have higher circulating levels of estradiol during the estrus stage of the cycle. Whether this trend will hold up for other stages of the cycle or for other mammotrophic hormones (progesterone and IGF-1) remains to be determined.

Reportable Outcomes: In progress.

Conclusions: The objective of this concept award is to determine whether the Sprague Dawley female rat can be used as a model to study the affect of onset of sexual maturation on breast cancer risk. Preliminary data is encouraging in that onset of VO in the rat is segregating with differences in body weight and circulating estradiol levels. The question of whether age at VO influences mammary gland biology as measured by changes in proliferation, differentiation or cell death or in response to exogenous hormones has yet to be determined.

References: None

Appendices: None